348

Determination of Added Rutin in Urine

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Introduction

The use of rutin, 5,7,3′,4′-tetrahydroxyflavonol-3-rutinoside, in the treatment of increased capillary fragility sometimes associated with hypertension (1), and in the treatment of certain other pathological conditions related to bleeding and capillary wall strength (2,3,4,5), has been reported in the literature. These clinical and pharmacological studies have indicated the desirability of precise methods for the determination of rutin in urine, and in other biological fluids and tissues. This paper describes a method for the determination of added rutin in urine.

Absorption in the ultraviolet spectrum (6) was not applicable, since interfering materials normally present in urine could not be satisfactorily separated from the rutin. The borocitric complex of Wilson *et al.* (7) and the fluorimetric method of Glazko *et al.* (8), based on the same reaction, were eliminated because of the difficulty of transferring the rutin from the urine into acetone and obtaining the resultant solution in an anhydrous condition.

The $FeCl_3$ reaction was not found useful because of its sensitivity to concentration, pH, and other factors. A search was made for other metal salts which with rutin might produce characteristic absorption in the visible spectrum more favorable to the establishment of a quantitative spectrophotometric method of analysis. Of the twenty-odd salts tested, the most satisfactory was $AlCl_3$, which with rutin produced a relatively stable intense yellow color with a definite absorption maximum at 413 mμ.

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Experimental

Reagents

(a) Aluminum chloride, 0.1 *M*—aqueous solution; (b) ammonium hydroxide, 1 part conc. NH_4OH plus 3 parts water; (c) potassium acetate, 1 *N*—aqueous solution; (d) acetic acid, glacial.

Apparatus

(a) Round-bottom centrifuge tubes, 15 ml.; (b) centrifuge, about 2000 r.p.m. An angle-head type centrifuge did not produce sufficient separation. By changing to a regular clinical centrifuge, the gel could be separated; (c) spectrophotometer or filter photometer, with cell compartment for 5 cm. cells.

Procedure

Preservation and Preparation of Urine Sample. It is convenient to use toluene as the preservative in collecting 24 hr. specimens of urine. However, before the sample for analysis is removed, as much of the toluene as is practical should be removed, so that suspended toluene mechanically carried along by the $Al(OH)_3$ gel does not later produce an error in the spectrophotometric determination. To separate the toluene, about 50 ml. of the 24 hr. specimen is with drawn from the original container by suction through a glass tube having a small orifice. By applying a small vacuum, any emulsion present is broken, and a clear solution is obtained.

Separation of Rutin from the Urinary Pigments. Place 2 ml. of urine in a 15-ml. round-bottom centrifuge tube. Add 3 ml. of 0.1 *M* $AlCl_3$ solution, mix, add 0.5 ml. of NH_4OH (1:3), mix, and dilute to about 10 ml. Centrifuge for 5 min. at 2000 r.p.m. decant the supernatant liquid, and break up the $Al(OH)_3$ gel by briskly tapping the middle of the tube against the finger. Add about 2 ml. of water and continue to break up the gel. Add one drop of the NH_4OH solution, mix, and dilute to about 10 ml. Centrifuge, and repeat the washing procedure. Decant the final wash solution and drain the tube for 20 to 30 sec. Add 0.1 ml. of glacial acetic acid by running it down the side of the tube and break up the gel as before. Stopper the tube and let stand overnight at room temperature to dissolve the gel.

Buffering, Development of Color, and Spectrophotometric Determination. Add 10 ml. of 1 *N* potassium acetate to the solubilized gel, mix, transfer to a 50-ml. volumetric flask, and make to volume with water. After about 30 min., and not more than 2 hr., determine the optical density of the solution at 413–416 mμ in a 5 cm. cell *vs.* a reagent blank carried through the above-described procedure.

Preparation of Standard Curve. Pipette a volume of each standard solution corresponding to 0.020, 0.050, 0.100, 0.150, and 0.200 mg. of the glycoside, into the round-bottom centrifuge tubes. Follow the above-described procedure exactly and plot optical density *vs.* the corresponding weight of rutin taken for analysis.

Calculations. From the standardization curve, determine the weight of rutin in the aliquot of urine taken for analysis, divide by the volume of the aliquot, and report as mg./ml. To report as mg./24 hr., multiply the mg./ml. by the volume for the 24 hr. specimen.

Discussion

The absorption curves for the rutin-aluminum chloride complex and for pure rutin are shown in Fig. 1. The wave length of the maximum is constant if the potassium acetate buffer and 0.1 ml. of glacial acetic acid are used (approximately pH 5.5), but shifts with relatively large changes in pH. Ethanol, if present in relatively large percentages, also shifts the wavelength of the maximum. Aluminum sulfate cannot be substituted for aluminum chloride.

Two to 5 ml. of urine may be used, depending on the concentration of rutin present. With larger volumes of urine, the amount of phos-

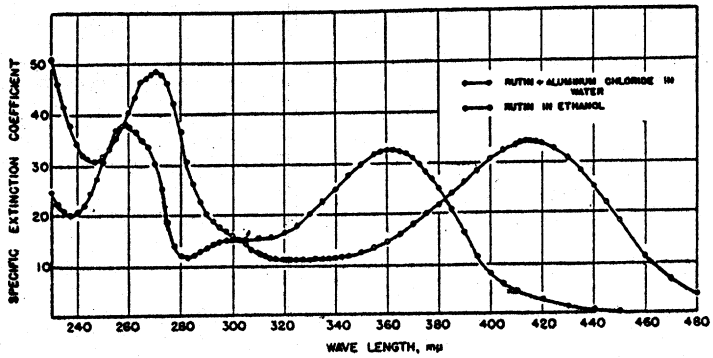

Fig. 1. Absorption spectra for rutin in ethanol and for the rutin-aluminum chloride complex in water. The specific extinction coefficients for both curves are based on 1 g. of rutin/l. in a 1 cm. cell.

phates precipitated by the reagents is increased. This increased quantity cannot be redissolved in the glacial acetic acid, and a significant error is introduced.

Solution of the aluminum hydroxide gel by 0.1 ml. of glacial acetic acid did not appear to be complete at the end of 6 hr. but was complete in 18 hr. Incomplete solution in all samples which had stood for 6 hr. or less was indicated, after addition of the buffer, by a sedimentation of $Al(OH)_3$ containing most of the rutin. Dilute acetic acid did not give complete solution even in 24 hr. Heat decreased the time required for solution, but the results obtained were erratic. Larger volumes of acetic acid lowered the final pH and produced lower optical densities, which could not be raised by further additions of potassium acetate.

Furthermore, the rate of solution was not significantly different even when 5 times as much acetic acid was used.

After addition of the potassium acetate buffer, the color intensity increased for about 30 min., was constant for approximately 3 hr., and then slowly faded over a period of 24 hr. All absorption measurements reported in this paper were made at some time between 30 min. and 1 hr.

The spectrophotometric measurements were made on a General Electric automatic recording spectrophotometer with a reagent blank. A straight line, which passed through the origin, was obtained for the standard curve.

TABLE I

Standardization Data. Rutin-Aluminum Chloride Complex vs. Reagent Blank

Sample	C[a] Wt. rutin	D[b] Optical density	D/C[c]
	mg.		
1	0.223	0.716	3.211
2	.185	.593	3.205
3	.156	.503	3.224
4	.130	.415	3.192
5	.089	.284	3.191
6	.074	.236	3.189
7	.022	.070	3.182
8	.019	.060	3.180

[a] C = mg. of rutin in a 2 ml. aliquot.

[b] D = optical density at 416 mμ, as measured in 5 cm. cells, of a solution containing the weight of rutin shown under "C" diluted to 50 ml.

[c] $10 \times D/C$ = specific extinction coefficient.

The rutin used for these experiments was purified by repeated alternate recrystallizations from absolute ethanol and water. The purified rutin was dried and weighed according to the methods described in a previous paper (6). The dried rutin (25 mg.) was dissolved in 3 ml. of absolute ethanol and diluted to 250 ml. with water. Further dilutions of this solution to obtain the concentrations indicated were also made with water. The analytical data for the standardization are shown in Table I.

In the recovery experiment presented in Table II, the dried rutin was dissolved in 3 ml. of absolute ethanol and diluted to 250 ml. with urine. Subsequent dilutions were made with urine to obtain the desired

TABLE II

Recovery of Rutin Added to Urine

Sample number	Wt. of rutin added to 2 ml. urine A	Total rutin determined per 2 ml. urine B	Added rutin recovered per 2 ml. urine[a] C	Difference $D = C - A$	Average per cent error $\frac{D}{A} \times 100$
	mg.	*mg.*	*mg.*	*mg.*	
Control	0.000	0.007	—	—	—
Control	.000	.007	—	—	—
1	.205	.221	0.214	+0.009	
2	.205	.220	.213	+ .008	+4.2
3	.144	.157	.150	+ .006	
4	.144	.155	.148	+ .004	+3.5
5	.103	.114	.107	+ .004	
6	.103	.114	.107	+ .004	+3.9
7	.041	.050	.043	+ .002	
8	.041	.048	.041	.000	+2.4
9	.021	.027	.020	− .001	
10	.021	.029	.022	+ .001	0.0

[a] The value for added rutin was obtained by subtracting the amount of rutin in the control from the total rutin determined in each sample ($C = B - 0.007$).

concentrations. Recoveries of added rutin have always been within 5% of theory.

To test the applicability of this method to the determination of rutin excreted in urine, a number of experiments were conducted. Four human subjects took by mouth quantitites of rutin ranging from 60 to 2250 mg. a day for 7–14 days. Analyses of the urine excreted by these subjects after variable periods revealed little or no rutin. The quantities detected in 24 hr. samples were so small that they were considered negligible. This was an unexpected finding, especially in view of the reports of Fukuda (9), Fukuda and Kono (10), Kono (11,12), and Garino (13), which indicated a rapid and complete excretion of flavonol glucosides administered to laboratory animals. In some of their experiments rutin was administered intravenously and in considerably larger quantities than were given to our subjects. It is possible that the dose and method of administration may affect the excretion of the glucoside. This problem is being investigated.

Compounds related to rutin, such as quercetin and quercitrin, interfere since they give a similar complex with aluminum chloride having somewhat different absorption curve shapes and maxima.

Summary

A relatively simple method is described for the determination of rutin added to urine. It is based on the color intensity of the complex formed by a buffered solution of rutin and aluminum chloride. Although it is not specific for rutin, the method gives quantitative recoveries of rutin added to urine.

Rutin could not be recovered in more than traces from the urine of 4 human subjects who had taken by mouth quantities of 60–2250 mg. a day for 7–14 days.

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